

Ribonucleolytic activities in the *Escherichia coli* in vitro translation system and in its separate components

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Abstract mRNA stability is a limiting parameter for the efficiency of in vitro protein biosynthesis. In order to develop strategies to prolong the mRNA half-life, we investigated the ribonuclease activities in the complete *Escherichia coli* system, in the separate cell fractions 70S ribosomes and S-100 and in the non-cellular fraction. Our results imply that the amount of ribonucleolytic activities and the insensitivity to placental RNase inhibitor in the complete system are due to the 70S ribosome fraction, whereas the generation of small degradation products is due to the S-100 fraction. Remarkably, the human placental RNase inhibitor is able to reduce mRNA degradation in the bacterial S-100 fraction.

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Key words: Cell-free protein synthesis; mRNA degradation; Endoribonuclease; Exoribonuclease; Cell fraction; Placental ribonuclease inhibitor

1. Introduction

Cell-free protein synthesis is a useful tool for the production of cytotoxic, regulatory or unstable proteins which cannot be (over)expressed in living cells [1]. Further advantages of in vitro translation are the synthesis of only one desired product, the possibility of facilitated and accelerated product isolation procedures by the use of peptide tags [2,3] and the capability of directed incorporation of modified amino acids [4,5]. Various in vitro translation systems were established based on components of prokaryotic or eukaryotic organisms and persistent efforts were made to increase their protein yields.

Our *Escherichia coli* in vitro translation system [6] consists of amino acids, energy providing factors, buffer, salts, several additional reactants, *E. coli* tRNAs, *E. coli* initiation factors, mRNA and *E. coli* 70S ribosomes and S-100. In such a reaction mixture the mRNA is degraded by ribonuclease activities associated with the cell fractions. Since mRNA stability is one of the major parameters limiting the efficiency of in vitro protein synthesis, it is very important that an improved in vitro translation system with increased mRNA half-life is developed.

In this study we investigate the degradation of the mRNA encoding the ribosomal protein L18 of *E. coli* during in vitro translation and the influence of the human placental RNase inhibitor. Based upon the results obtained new strategies are discussed for prolonging the half-life of mRNA.

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Abbreviations: BSA, bovine serum albumin; DEPC, diethylpyrocarbonate; DTE, dithioerythritol; RI, ribonuclease inhibitor; TCA, trichloroacetic acid

2. Materials and methods

2.1. In vitro transcription

The radiolabelled mRNA transcript was obtained by in vitro transcription of the *Hind*III-linearized plasmid pKK504-3 carrying the gene for the protein L18 with T3 RNA polymerase following the protocol of Triana-Alonso et al. [7] with some modifications. The reaction mixture, which was incubated for 120 min at 37°C, contained 40 mM Tris-HCl (pH 8.1), 22 mM MgCl₂, 20 nM plasmid DNA, 5 mM DTE, 0.1 µg/µl BSA, 1 mM spermidine, 1 U/µl human placental RNase inhibitor (Boehringer Mannheim), 2 U/µl T3 RNA polymerase (Boehringer Mannheim), 3.75 mM ATP, GTP and UTP, whereas the concentration of CTP was 2.5 mM only, and [α -³²P]CTP (Amersham) with a specific activity of 3.3 µCi/pmol was added to 5 µCi/µl. Then the transcription mixture was treated with 1 U/µl DNase I (Boehringer Mannheim) for 20 min at 37°C, incubated for 4 min at 55°C and cooled down to 35°C within 120 min. Afterwards the rena-tured mRNA transcript was isolated by phenol, chloroform and diethylether extraction and purified by gel filtration with fractionating elution using a NICK column (Pharmacia). The peak fractions were pooled and the RNA concentration was determined at 260 nm. The integrity of the mRNA was analyzed by denaturing electrophoresis on a 6% polyacrylamide/7 M urea gel [8].

2.2. In vitro translation

The cell-free protein synthesis reaction was performed according to Baranov and Spirin [9] applying 150 nM radiolabelled pKK504-3/*Hind*III transcript as mRNA template. Human placental RNase inhibitor was added to a final concentration of 0.1 U/µl.

The preparation of 70S ribosomes and S-100 from *E. coli* D10 (*relA1*, *spoT1*, *metB1*, *RNase I*) [10] was carried out as described by Gold and Schweiger [11] except that the ribosomes were ultracentrifuged in the presence of 500 mM ammonium chloride.

2.3. mRNA decay assays

150 nM purified and radiolabelled mRNA was incubated in the entire in vitro translation mixture or in incomplete mixtures lacking either 70S ribosomes or S-100 or both. For electrophoretic analysis aliquots of 15 µl were removed after 0, 10, 20, 30, 40, 50, 60 min of incubation, diluted with 45 µl ice-cold DEPC-treated water, extracted successively with 60 µl phenol and 60 µl chloroform and deproteinized by treating the samples with 500 ng/µl proteinase K (BRL) for 3 min at 60°C. Their Cerenkov radiation was determined and the mRNA and its degradation products were analyzed by denaturing electrophoresis as described in Section 2.1. After autoradiography the bands of intact mRNA were cut out of the gel and their Cerenkov radiation was measured. For TCA precipitation two samples, 10 µl each, were taken out of the reaction mixture at the same time points as for the

Table 1

	Full-length mRNA half-life (min)
Complete system+RI	4.9
Complete system−RI	4.7
70S ribosomes+RI	10.7
70S ribosomes−RI	10.0
S-100+RI	41.3
S-100−RI	18.1

Comparison of effects of cell fractions and RNase inhibitor (RI) on full-length L18 mRNA half-life calculated from Fig. 1. More details are given in the text.

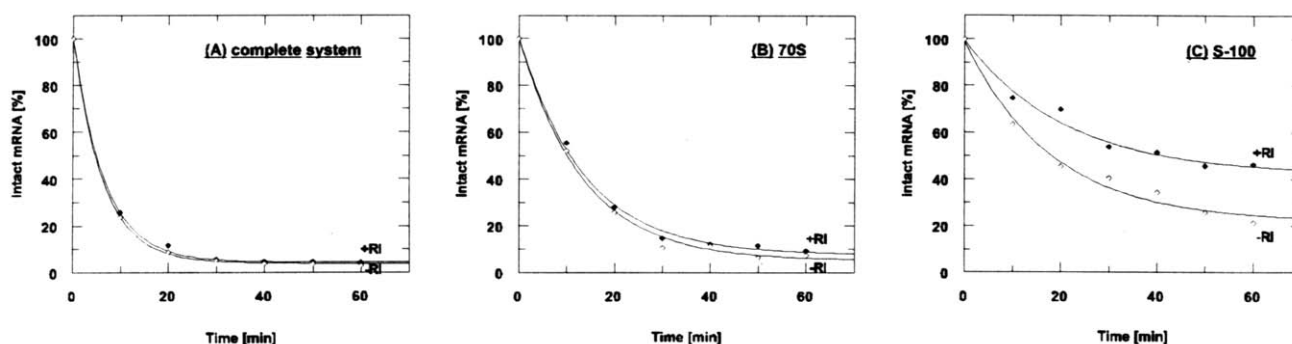


Fig. 1. Comparison of effects of the cell fractions on degradation of full-length mRNA analyzed electrophoretically by measuring the radioactivity of the bands of intact mRNA. Time course of mRNA decay (A) in the complete in vitro translation system, (B) in the partial system with the non-cellular components and 70S ribosomes and (C) in the partial system with the non-cellular components and S-100, each with (♦) or without (◇) RNase inhibitor.

electrophoresis. The mRNA and its degradation products longer than about 50 nt were precipitated with 5% TCA [12] and quantified by Cerenkov counting.

3. Results

The mRNA transcript used in our experiments has an overall length of 407 nt consisting of the 29 nt long non-coding 5'-terminal region, including the translation initiation signals, and the sequence encoding protein L18. The molecule lacks an untranslated 3'-terminal segment and the immediate 3'-end is constituted by the stop codon.

The full-length mRNA decay kinetics in the complete *E. coli* in vitro translation system (Fig. 1A) shows a considerable mRNA degradation process with an insignificant protective efficacy of RNase inhibitor, similar to the course of mRNA decay in the partial system with 70S ribosomes (Fig. 1B). Nevertheless, in the case of 70S ribosomes, the degradation is somewhat less and the effect of RNase inhibitor is slightly increased. Contrarily, the partial system with S-100 shows a quite different course of mRNA decay (Fig. 1C, Table 1) with an extended mRNA half-life and a significant protection by RNase inhibitor. Compared with the partial systems with the separate cell fractions (Fig. 2B,C), the complete in vitro translation system (Fig. 2A) yields a large quantity of small mRNA degradation products. Corresponding to the full-length mRNA decay kinetics only the partial system with S-100 shows a significant influence of RNase inhibitor on the amount of the TCA-precipitable mRNA portion.

Summarizing the results leads to a scheme of mRNA prod-

ucts (Fig. 3), reflecting the effects of the cellular and non-cellular components on mRNA turnover (Fig. 4). The main ribonuclease activities occur in the cell fractions. The non-cellular components contain very little RNase activity, which is almost totally inhibitable by the RNase inhibitor. The results suggest that quantity and insensitivity to RNase inhibitor of the mRNA degradation process in the complete in vitro translation system are dominated by the 70S ribosome fraction while the capacity to generate small degradation products is predominantly provided by the S-100 fraction.

4. Discussion

The mRNA degradation process in the complete in vitro translation system is due to the interaction of both cell fractions combining the intensive, hardly inhibitable ribonuclease activity of 70S ribosomes with the highly processive capacity of S-100. Obviously, this co-operation is in accordance with the general model for the mechanism of mRNA degradation in prokaryotes describing a concerted action of endonucleases and processive 3' → 5'-exonucleases [13,14]. Our results agree with previous findings showing that the prevalent 3' → 5'-exonucleases involved in mRNA degradation in *E. coli*, RNase II and PNPase [15], are mainly found in the soluble cell fraction [16,17]. Such exoribonucleolytic activities give rise to the loss of coding sequence including the stop codon, entailing incomplete, possibly functionally truncated proteins and in vivo selective proteolysis [18]. Hence, the most important step to prolong mRNA half-life is the impairment of the 3' → 5'-exonucleases, for example by 3'-terminal, protective structural

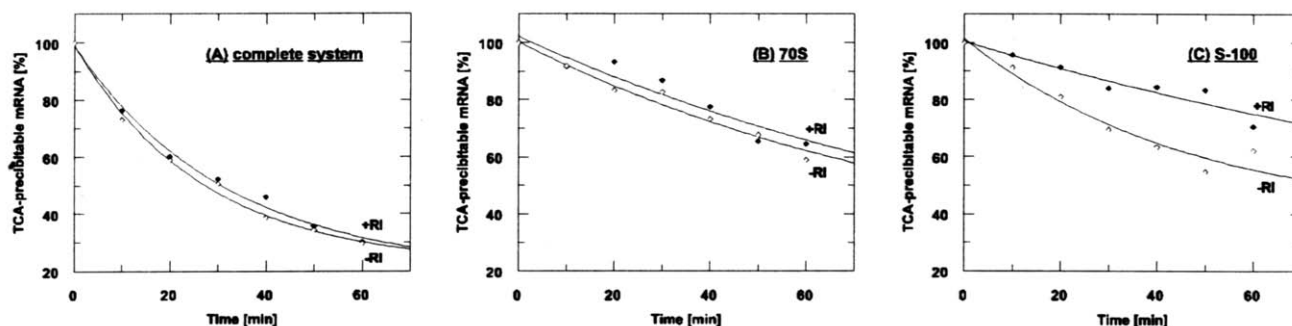


Fig. 2. Comparison of effects of the cell fractions on formation of small mRNA degradation products (< 50 nt) analyzed by measuring the radioactivity of the TCA-precipitable mRNA portion (full-length mRNA and fragments > 50 nt). For graph designation see Fig. 1.

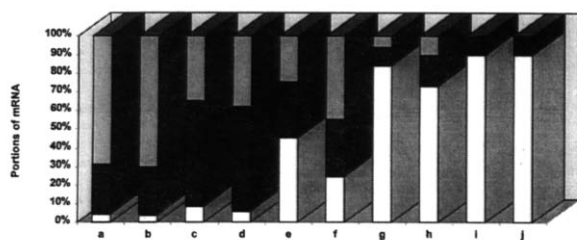


Fig. 3. Comparison of effects of the separate components on quality and quantity of mRNA degradation products summarizing the electrophoretic analysis and the TCA precipitation assays. Portions of intact mRNA (full-length mRNA band, white bars), long mRNA degradation products (TCA-precipitable portion minus intact mRNA, fragments > 50 nt, black bars) and small mRNA degradation products (TCA-soluble portion, fragments < 50 nt, gray bars) after 60 min of incubation in the complete in vitro translation system with (a) or without (b) RNase inhibitor, in the partial system with the non-cellular components and 70S ribosomes with (c) or without (d) RNase inhibitor, in the partial system with the non-cellular components and S-100 with (e) or without (f) RNase inhibitor, in the non-cellular components with (g) or without (h) RNase inhibitor and as the control in DEPC-treated water with (i) or without (j) RNase inhibitor.

barriers like REP sequences and intrinsic terminators ([19], our laboratory, unpublished results). Since *E. coli* strains deficient in RNase II, PNPase, RNase E, RNase III or RNase I are available, further potential to diminish the amount of ribonucleolysis is given by the use of appropriate bacterial strains for isolation of cell fractions and the modification of their preparation procedures.

The poor protective efficacy of RNase inhibitor in the complete in vitro translation system is consistent with its insignificant effect on protein yields (unpublished results). On the

other hand, the human RNase inhibitor is able to reduce the ribonuclease activities in the non-cellular components and in the S-100 fraction of *E. coli*.

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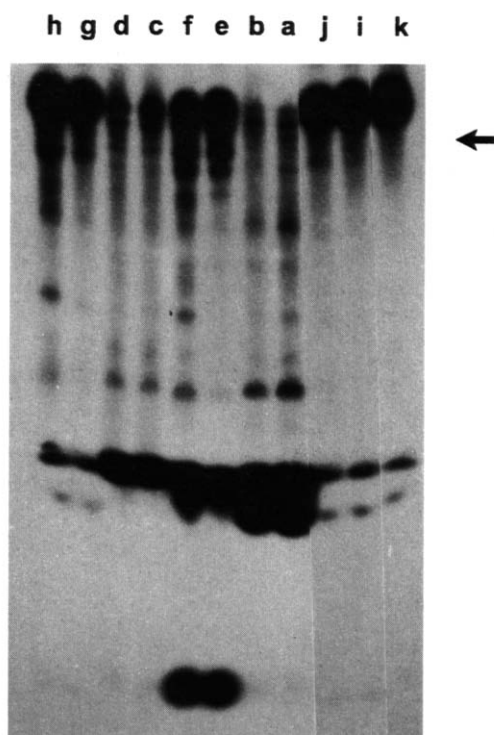


Fig. 4. Autoradiogram of the mRNA (lane k, arrow) and its degradation products after 60 min of incubation. For lane designation see Fig. 3.